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FKBP22 is part of chaperone/folding catalyst complexes in the endoplasmic reticulum of *Neurospora crassa*

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Abstract FKBP22 is a dimeric protein in the lumen of the endoplasmic reticulum, which exhibits a chaperone as well as a PPIase activity. It binds via its FK506 binding protein (FKBP) domain directly to the Hsp70 chaperone BiP that stimulates the chaperone activity of FKBP22. Here we demonstrate additionally the association of FKBP22 with the molecular chaperones and folding catalysts Grp170, α-subunit of glucosidase II, PDI, ERp38, and CyP23. These proteins are associated with FKBP22 in at least two protein complexes. Furthermore, we report an essential role for FKBP22 in the development of microconidiophores in *Neurospora crassa*.

Keywords: Chaperone; FKBP; BiP; PPIase; Disulfide isomerase; Endoplasmic reticulum

1. Introduction

The endoplasmic reticulum (ER) is specialized for the folding of proteins designated for secretion, membranes, or organelles. It provides some unique conditions for the folding of these proteins like disulfide bond formation, protein glycosylation, and a stringent quality control [1,2]. Two major chaperone systems in the ER have been studied intensively, the calnexin/calreticulin system and the BiP system. The expression of these chaperones is induced as response to elevated levels of unfolded proteins in the ER the so-called unfolded protein response (UPR) [3]. Both chaperone systems recognize unfolded proteins and promote their folding by cycles of substrate binding and release. The calnexin/calreticulin system depends on N-linked monoglucosylated oligosaccharides that serve as a binding site for the chaperones [4]. Therefore, calnexin and calreticulin are also called lectin chaperones. The second chaperone system depends on recognition of unfolded hydrophobic regions on proteins by the Hsp70 chaperone BiP [5]. BiP and the lectin chaperones are involved in the folding of proteins in the ER and they seem to act independent from each other. The decision, which of the two systems guides a substrate protein on the way to its native conformation, seems to be a fine tuned process and depends on the nascent protein itself. It seems to take place already during translocation of the nascent proteins into the ER [6]. Some substrate proteins are processed sequentially by both chaperone systems, others bind calnexin and calreticulin but not BiP and some interact exclusively with BiP (e.g. [7–9]). Beside chaperones folding catalysts like disulfide isomerases and peptidyl prolyl *cis–trans* isomerases (PPIases) are involved in protein folding in the ER [10]. PPIases, like FK506 binding proteins (FKBPs) or cyclophilins (CyPs), catalyze the *cis–trans* isomerization of Xaa–Pro bonds in oligopeptides and proteins, a time limiting step in folding of certain proteins.

There is increasing evidence that chaperones and folding catalysts in the ER act together by forming complexes to fold nascent proteins and several examples of substrate proteins, which are associated with chaperones and folding catalysts, are reported. The chaperone and folding catalyst composition varied between these complexes, possibly reflecting the different folding requirements of the different substrates (e.g. [11,12]). Interestingly, the formation of these chaperone/folding catalyst complexes seems to be independent on the presence of a nascent substrate protein [13]. It was supposed that these complexes are forming a chaperone network in the ER that promotes folding of proteins and ensures protein quality control.

We recently identified the dimeric FKBP22, a PPIase from *Neurospora crassa*, as a novel chaperone in the ER [14]. FKBP22 is directly associated with BiP and both proteins are forming a complex with higher chaperone activity than the single proteins alone. In this study we report a growth defect for a FKBP22 deletion mutant strain and identify by chemical cross-linking a number of chaperones and folding catalysts that are specifically associated with FKBP22.

2. Material and methods

2.1. Growth of *N. crassa*

Wild type *N. crassa* strain 74-OR23-1A (WT) and strain ΔFKBP22 were grown in 0.5% saccharose (Sacc) and 2% carboxylmethylcellulose (CMC) supplemented with Vogels medium at 25 °C for 18–20 h in

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Abbreviations: ER, endoplasmic reticulum; UPR, unfolded protein response; SEC, size exclusion chromatography; DSP, dithiobis(succinimidyl propionate); PI, preimmuneserum; CMC, carboxylmethylcellulose; Sacc, saccharose; MM, molecular mass; PPIases, peptidyl prolyl *cis–trans* isomerases; FKBP, FK506 binding protein; CyP, cyclophilin

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liquid culture as described [15]. Media supplemented with 15 g/l agar, Vogels, and 2% CMC or 2% Sacc were used for growth in petri dishes. Where indicated, the medium was supplemented with 0.5 mM H_2O_2.

2.2. Protein identification/computational analysis

Mass analysis was done at Toplab, Martinsried, Germany. Homologous protein search was performed with Basic Local Alignment Search Tool [16]. Conserved motifs were identified using the Conserved Domain Database [17]. Predicted proteins were identified based on the published *N. crassa* genome [18].

2.3. Miscellaneous

Subcellular fractionation, Immunoprecipitation, and size exclusion chromatography (SEC) were performed as described [14]. Disruption of FKBP22 in *N. crassa* (ΔFKBP22) was performed by repeat-induced point-mutations, as described previously [19]. Nuclei in conidia were stained with 4′,6-diamidino-2-phenylindol (DAPI), as described previously [20].

3. Results and discussion

3.1. A FKBP22 deletion mutant of N. crassa has a defect in forming microconidiophores

FKBP22 has a typical PPIase activity, functionally cooperates with BiP as a chaperone [14,15]. It therefore seemed likely that FKBP22 is part of the ER protein folding machinery of *N. crassa*. To investigate in vivo functions of FKBP22, the phenotypes of an FKBP22 deletion strain (ΔFKBP22) and the WT strain were compared. ΔFKBP22 is viable on a variety of media/temperatures and has no general growth defect (data not shown). This observation is in agreement with reports of other PPIase mutants. For example, the deletion of all FKBPs and CyPs in *Saccharomyces cerevisiae* resulted in no obvious growth defect [21]. On the other hand, FKBPs and CyPs are highly conserved and distributed in all organelles. Thus, PPIases seem to fulfill specific functions on a restricted number of partner proteins. Even though deletion of FKBP22 is not lethal to *N. crassa*, we observed a growth defect of ΔFKBP22 in developing conidiophores at the inoculation point during growth on nutrient limited conditions (Fig. 1A). *N. crassa* forms two types of conidiophores during the asexual development [22]. Macroconidiophores produce macroconidias, which are 5 μm long and contain several nuclei ranging from 3 to 20 per conidium. In contrast, microconidiophores produce microconidia, which are approximately 2 μm long and uninucleate. To identify which kind of conidia are produced at the inoculation point, nuclei from conidia were stained with DAPI. As control, conidia collected from conidiophores at the edge of the petri dish were stained. Most (95%) of the conidia from the inoculation point were uninucleate and smaller than the conidia collected from the edge of the petri dish (Fig. 1B). They are therefore microconidia and the observed conidiophores were microconidiophores. Little is known about microconidiation in *N. crassa*, but it occurs best in media with low concentrations of ammonium nitrogen and sugar, supplemented with cellulose [22]. This is in agreement with our observations, since we observed formation of microconidiophores on CMC medium, while it was repressed on Sacc medium (Fig. 1A). Interestingly, addition of H_2O_2, which causes oxidative stress, induced the aerial hyphae formation both in WT and ΔFKBP22 strain. Reducing conditions, caused by β-mercapthoethanol, repress microconidiophore formation in WT and ΔFKBP22 strains (data not shown). Therefore, ΔFKBP22 is able to form microconidiophores and only the initiation seems to be disturbed on CMC medium. At the beginning of the morphogenetic transition from mycelium to aerial hyphae a hyperoxidant state is developed in *N. crassa*, and this hyperoxidant state was suggested to trigger the morphogenetic transition [23]. Thus, it is likely that the oxidative stress caused by addition of H_2O_2bypasses the defect caused by the absence of FKBP22 and triggers the ΔFKBP22 strain to form microconidiophores like Wt strain. However, the exact role of FKBP22 in this morphogenetic transition is object of further investigations.

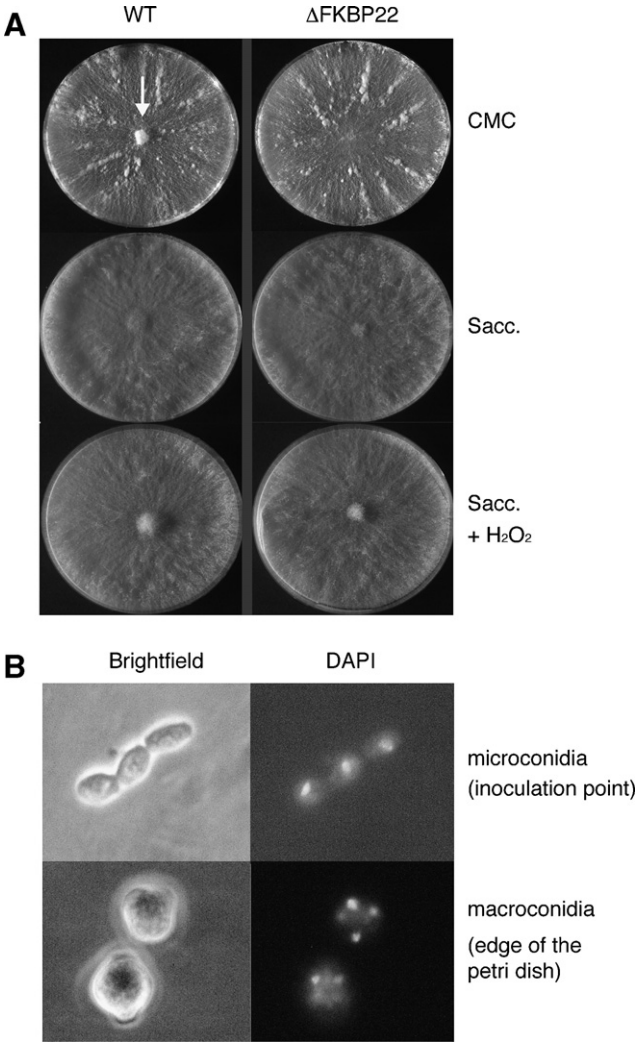

Fig. 1. ΔFKBP22 strain has a defect in developing microconidiophores at the inoculation point. (A) Agar plates where inoculated in the center (as indicated by the arrow on plate WT/CMC) with a 10 μl of a conidia suspension (10^8/ml) from WT and ΔFKBP22 as indicated. Medium was supplemented with 0.5 mM H_2O_2 as indicated (CMC = carboxylmethylcellulose, Sacc. = saccharose). Photographs were taken after 50 h. (B) Nuclei staining of WT conidia collected from conidiophores from the inoculation point and the edge of the petri dish.

3.2. FKBP22 is associated with several chaperones and folding catalysts in the endoplasmic reticulum

BiP has been shown to be part of chaperone/folding catalyst complexes, and we were interested in the involvement of FKBP22 in such complexes. To identify novel binding partners,

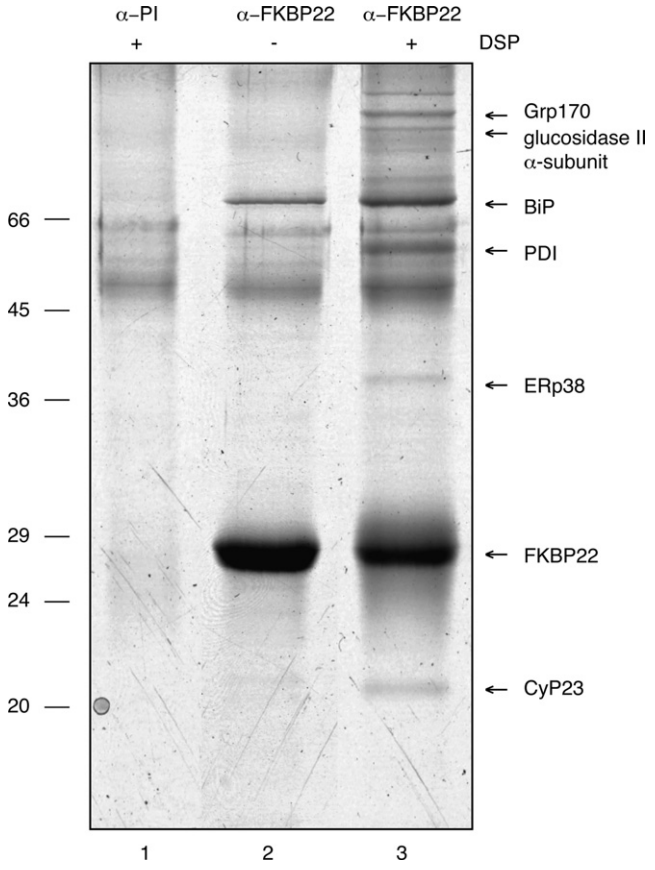

Fig. 2. FKBP22 is associated with several chaperones and folding catalysts. WT microsomes (15 mg) were treated with 150 μg/ml DSP (lanes 1/3) as indicated before lysing with 0.5% NP-40. Proteins were precipitated with αFKBP22 antiserum (lanes 2/3), or preimmuneserum (PI) (lane 1) and protein A Sepharose. Immunoprecipitated proteins were separated by SDS–PAGE, Coomassie stained and identified by mass spectrometry.

FKBP22 was isolated by immunoprecipitation from the microsomal fraction of *N. crassa*, which was treated with the membrane permeable and thiol-cleavable cross linker dithiobis(succinimidyl propionate) (DSP) to stabilize protein interactions (Fig. 2). The isolated proteins were subsequently identified by mass spectrometry (Table 1). BiP was the only protein co-immunoprecipitating without DSP [14] (Fig. 2, lane 2). In DSP treated microsomes, five additional proteins were co-immunoprecipitated routinely (Fig. 2, lane 3). These proteins specifically bound to FKBP22 because they were absent in controls (Fig. 2, lane 1). The amount of co-precipitated BiP was increased in the presence of the cross linker and it was the most prominent protein co-immunoprecipitating with FKBP22. According to the identification by mass spectrometry, the 60 kDa band represents PDI, the most abundant disulfide isomerase in the ER. Furthermore, the Hsp70 chaperone Grp170, the disulfide isomerase ERp38, and CyP23, a PPIase of the CyP type, were identified. Surprisingly, we co-isolated also the α-subunit of glucosidase II. Although glucosidase II is intimately linked, together with calnexin and calreticulin, to the maturation of glycoproteins in the ER [4], it has so far never been found in a complex with ER resident chaperones. However, in *S. cerevisiae* a synergistically function of BiP and the glucosidase II has been observed [24], and it seems conceivable that the enzyme acts on glycoproteins, which require BiP or other proteins of the chaperone/folding catalyst complex for proper folding. In the immunoprecipitation no BiP regulating co-chaperones were detected. To bind and to release a substrate, BiP has to be regulated by co-chaperones. There are reports of Hsp40 proteins associated with BiP and other folding helpers in the ER [13]. However, in mouse lymphoma cells the Hsp40 Erdj3 is present in a chaperone/folding catalyst complex only in presence of a substrate [13]. Therefore, the regulating co-chaperones might be recruited to the complex only in the presence of a substrate. In yeast it has been shown that the homologues of BiP and Grp170 stimulate each others ATPase activity [25]. It is, therefore, also conceivable that Grp170 stimulates the substrate release from BiP.

All proteins co-immunoprecipitated with FKBP22 are chaperones or folding catalysts. They seem to form one or several chaperone/folding catalyst complex/es in the ER of *N. crassa*, similar to complexes that have been described for other organisms (e.g. [11–13]). While homologues of Grp170, PDI, ERp38, and CyP23 have previously been shown to be associated with BiP or with BiP/substrate complexes, FKBP22 is the first FKBP found in such complexes.

3.3. Associations between chaperones and folding catalysts

In order to investigate the interactions between these folding helpers, specific antisera against recombinant *N. crassa* BiP, PDI, ERp38, and CyP23 were raised in rabbits (data not shown). We compared the relative amount of these five proteins in microsomes from WT and ΔFKBP22. Interestingly, the relative amounts were not altered in ΔFKBP22 when compared to the WT strain (Fig. 3A). Therefore, the deletion of FKBP22 does not result in high amounts of misfolded proteins, which would induce a UPR.

Table 1
Identification of proteins by mass spectrometry

Proteins identified	Sequence coverage (%)	Homologue protein[b]	Sequence[c] identity (%)	Conserved motif[d]
NCU04203.2[a]	31	Glucosidase II α-subunit	64	Glycosyl hydrolase familie 31
NCU09223.2[a]	38	PDI	80	Thioredoxin domain
Grp170	59			Molecular chaperones GRP 170/SIL1
BiP	52			HSP70 domain
ERp38	80			Thioredoxin domain
CyP23	45			Cyclophilin

[a]Proteins were identified as putative proteins from the Neurospora Genome Sequencing Project [18].
[b]Homologues to the putative proteins.
[c]Their sequence identity.
[d]Conserved sequence motifs were identified by computational analysis.

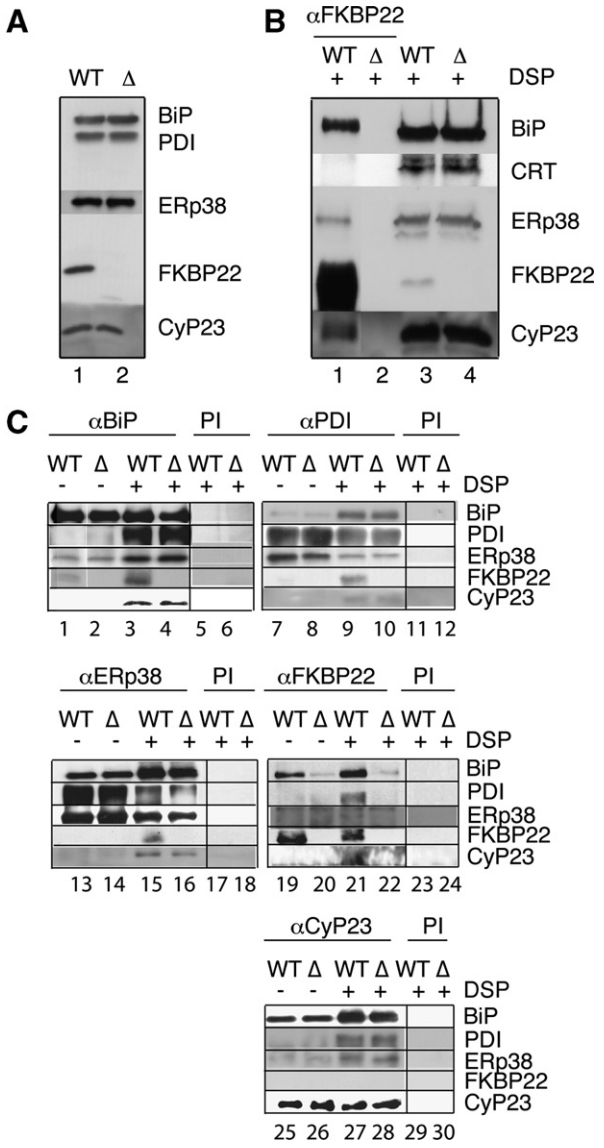

Fig. 3. Associations between chaperones and folding catalysts. (A) Levels of BiP, PDI, Erp38, and CyP23 are identical in WT and the ΔFKBP22 strain. Microsomes (15 μg) from WT (lane 1) and ΔFKBP22 (lane 2) were separated by SDS–PAGE and transferred to nitrocellulose membranes for Western analysis with the indicated antisera. (B) Cross linking is specific because calreticulin is not co-precipitating with FKBP22. Microsomes (3 mg) from WT (lane 1/3) and ΔFKBP22 (lane 2/4) were treated with 150 μg/ml DSP, and proteins were precipitated with αFKBP22 antiserum (lanes 1/2). As control, 15 μg of microsomal proteins were directly used for separation by SDS–PAGE and Western analysis with the indicated antisera (lanes 3/4). (C) The chaperones and folding catalysts form complex(es) in *N. crassa*. Antisera directed against BiP, PDI, ERp38, FKBP22, and CyP23 were used for immunoprecipitation from WT and ΔFKBP22 strain derived microsomal lysates (3 mg) as indicated. Preimmunserum was used as a control. Where indicated, microsomes were treated with 150 μg/ml DSP. The precipitated proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes for Western analysis with the indicated antisera.

To rule out the possibility of unspecific cross linking by DSP, the presence of the major lumenal ER protein calreticulin in the FKBP22 immunoprecipitation was investigated. While BiP, ERp38, and CyP23 were specifically associated with FKBP22, calreticulin was not co-precipitated (Fig. 3B). Therefore, cross-linking with DSP was specific and stabilizes protein interactions that are otherwise not stable to lysis of microsomes and immunoprecipitation.

Associations between the folding helpers were investigated by immunoprecipitations from microsomes, using antisera directed against BiP, PDI, ERp38, FKBP22, and CyP23. An interaction between following proteins was observed: PDI and ERp38 (Fig. 3C lane 7/8, 13/14), BiP and ERp38 (Fig. 3C lane 1/2, 13/14), BiP and CyP23 (Fig. 3C lane 25/26), BiP and FKBP22 (Fig. 3C lane 1, 19). BiP seems to play a central role in the organization of the folding helpers, since it is associated with three of the four proteins. After treatment of microsomes with DSP, all five proteins were precipitated, irrespective of which of the five antisera was used for immunoprecipitation. Only the antiserum directed against CyP23 did not co-immunoprecipitate detectable amounts of FKBP22, whereas all other components were present (Fig. 3C lane 27). This suggests the existence of a complex composed of all of these five proteins. The absence of FKBP22 in the ΔFKBP22 strain had no general influence on these associations, and, therefore, FKBP22 seems to have no function in the organization of the complex/es (Fig. 3C). The investigated folding helpers seem to be organized in subcomplexes stable enough to be detected by immunoprecipitation. Cross linking stabilizes the chaperone/folding catalyst complex/es, originally detected by immunoprecipitation of FKBP22 (Fig. 2).

To separate the identified complex/es by their native molecular masses (MMs), SEC was performed with microsomal lysate. FKBP22 in the single fractions was immunoprecipitated, and the presence of co-precipitated BiP, PDI, ERp38, FKBP22, and CyP23 was analyzed by Western analysis. We have previously shown that FKBP22 is present in two discrete peaks in SEC. A 50 kDa peak represents a free dimeric form and the 270 kDa peak represents the BiP/FKBP22 complex [14]. As expected, PDI, ERp38, and CyP23 were not associated with FKBP22 in the absence of the cross linker (Fig. 4A). After

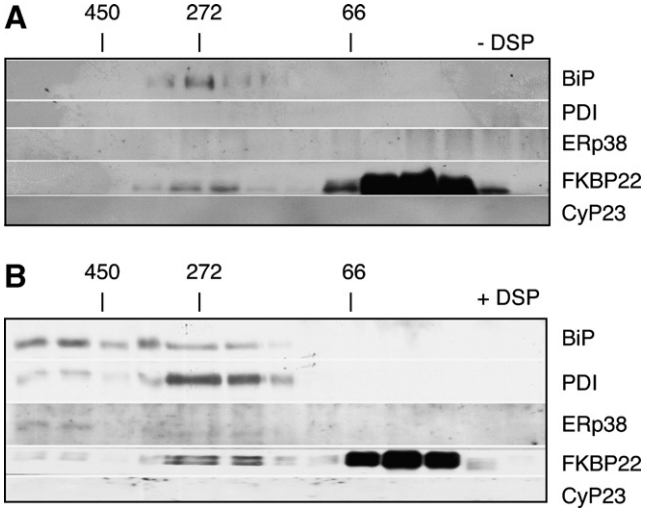

Fig. 4. FKBP22 is part of two chaperone/folding catalyst complexes. (A) Microsomes (1.5 mg) from WT strain were lysed with 0.5% NP-40 and separated by SEC. Proteins in the obtained fractions were immunoprecipitated with αFKBP22 specific antiserum, separated by SDS–PAGE, and analyzed by Western analysis with the indicated antisera. (B) As in A, with microsomes that were treated with DSP (150 μg/ml).

treatment with DSP, FKBP22 additionally appeared in higher MM fractions of approximately 500 kDa (Fig. 4B). BiP and PDI were associated with FKBP22 in the 270 kDa as well as in the 500 kDa fractions. While the majority of PDI was present in the 270 kDa fractions, the majority of BiP is present in the 500 kDa fractions. On the other hand, ERp38 is associated with FKBP22 only in the 500 kDa fractions. CyP23 was not co-isolated with FKBP22 in detectable amounts. These results suggest that FKBP22 is part of at least two chaperone/folding catalyst complexes with MM of 270 kDa and 500 kDa and that these complexes are composed of a distinct set of chaperones and folding catalysts. This distinct composition could reflect the requirements of different substrate proteins or different folding states of substrate proteins. Thus, the ER of *N. crassa* harbors chaperone/folding catalyst complexes, similar to those reported for rat, mouse, and human cells, possibly constituting an ER network as proposed recently (e.g. [11–13]).

4. Conclusions

In summary, we present data supporting the existence of novel chaperone/folding catalyst complexes associated with FKBP22 in the ER of *N. crassa*. The glucosidase II in this complex could provide a link between the BiP and the lectin chaperone system. FKBP22 is the first FKBP shown to be part of such complexes. Furthermore, we report an essential role for FKBP22 in the development of microconidiophores in *N. crassa*.

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